RH: SHORT COMMUNICATIONS

Long-Term Storage of *Cryptosporidium parvum* for In Vitro Culture

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Abstract: The long-term storage of *Cryptosporidium* life-cycle stages is a prerequisite for in vitro culture of the parasite. *Cryptosporidium parvum* oocysts, sporozoites and intracellular forms inside infected host cells were stored for 6 to 12 mo in liquid nitrogen utilizing different cryoprotectants (dimethyl sulfoxide [DMSO], glycerol and fetal calf serum [FCS]), then cultured in vitro. Performance in vitro was quantified by estimating the total *Cryptosporidium* copy number using qPCR in 3- and 7-day-old cultures. While only few parasites were recovered either from stored oocysts or from infected host cells, sporozoites stored in liquid nitrogen recovered from freezing successfully. More copies of parasite DNA were obtained from culturing those sporozoites than sporozoites excysted from oocysts kept at 4 C for the same period. The best performance was observed for sporozoites stored in RPMI with 10% FCS and 5% DMSO, which generated 240% and 330% greater number of parasite DNA copies (on days 3- and 7-post-infection, respectively) compared to controls. Storage of sporozoites in liquid nitrogen is more effective than oocyst storage at 4 C and represents a more consistent approach for storage of viable infective *Cryptosporidium* aliquots for in vitro culture.

In vitro culture is an essential tool to study the apicomplexan gut parasite *Cryptosporidium* (Karanis and Aldeyarbi, 2011), but despite recent advances in culture systems (Morada et al., 2016), long-term storage of isolates continues to depend on maintenance of oocysts at 4 C in a refrigerator. Production of oocysts in culture may be

sparse (Karanis and Aldeyarbi, 2011) and there are no methods for long-term storage of sporozoites or other short-lived stages. We present here observations on long-term (6 mo and 1 yr) preservation of *Cryptosporidium* life cycle stages in liquid nitrogen, which offer an alternative approach to storage of this intractable pathogen.

Cryptosporidium first came to attention some 35 yr ago as an important human gut pathogen causing severe diarrhea (Bird and Smith, 1980), and is best known for the infection of several hundred thousand individuals in the Milwaukee incident in 1993 (MacKenzie et al., 1995). The huge number of oocysts produced by a diarrheic infected host, the apparent long life span of oocysts and the possibly severe consequences of infection, in particular in immunocompromised individuals, make this a highly significant pathogen, recognized by its classification by Centers for Disease Control and prevention as a potential biowarfare agent (www. emergency.cdc.gov/bioterrorism).

Calves are the natural hosts of *C. parvum*, while *C. hominis* has no natural host apart from humans and is therefore only available from clinical isolates. Neonatal or SCID mouse models for these pathogens require infectious doses 10-100 times higher than their natural hosts (see e.g., Fayer et al., 1991; Zambriski et al., 2013). With concerns over the validity of these experimental models, and the welfare and logistic issues surrounding maintenance in animal hosts, we are dependent on in vitro culture to advance our understanding of *Cryptosporidium* biology. Long-term storage is an essential adjunct of in vitro culture, partly because of the propensity for genetic drift in cultures or animal hosts, but also because oocyst production in culture is not yet as prolific as that from natural hosts (Karanis and Aldeyarbi, 2011), although long-term maintenance with substantial oocyst production has recently been described (Morada et al., 2016; DeCicco RePass et al., 2017). In general, in vitro cultures are initiated from oocysts stored at 4 C in PBS supplemented with antibiotics as collected from animal hosts, on the assumption that these stages are resistant and can survive for many

months, despite the experimental observation that oocyst survival declines sharply after 3 mo in these conditions (Liang and Keeley, 2012; Paziewska-Harris et al., 2016). Other apicomplexans, such as *Plasmodium*, are routinely cultured in vitro from isolates stored in liquid nitrogen for many months or years. Here we present encouraging results on the storage of *Cryptosporidium parvum* in liquid nitrogen for 6 mo and 12 mo using an alternative infectivity assay, which suggests that long-term storage in liquid nitrogen could become an important tool in *Cryptosporidium* in vitro culture.

Oocysts of *C. parvum* IOWA strain (Waterborne Inc., New Orleans, Louisiana) were stored prior to use at 4 C in PBS supplemented with 100U/ml penicillin, 100 µg/ml streptomycin, 10 mg/ml gentamicin, 0.25 µg/ml Amphotericin B and 0.01% Tween 20. The oocyst batch used was 4 mo old (post shedding by calves and purification) when storage experiments began. The concentration of supplied oocysts was initially calculated using a hemocytometer (W. Schreck,Hofheim/TS, Germany), and samples of appropriate numbers of oocysts for experiments were generated by dilution. Human colon adenocarcinoma (HCT-8; ATCC CCL 244) cells were maintained as described previously (Paziewska-Harris et al., 2015), and grown in 6-well plates (Thermo Fisher Scientific, Landsmeer, The Netherlands; 9.6 cm^2 per well) at 37 C in an atmosphere containing 5% CO_2 until they reached 90% confluence. Before storage or infection of host cells (in case of stored oocysts, see below), oocysts were excysted (Hijjawii et al., 2001) by incubation for 30 min at 37 C in a 0.25% trypsin (from bovine pancreas, Sigma, cat. no. T1426, Zwijndrecht, The Netherlands) solution (pH 2.5, adjusted with 1M hydrochloric acid), followed by centrifugation at 2000 × *g* for 5 min, after which the trypsin solution was replaced by *Cryptosporidium* maintenance medium (CMM; Hijjawi et al., 2001 as modified by Paziewska-Harris et al., 2015) containing 200 µg/ml of bile salts (from bovine and ovine pancreas, Sigma, cat. no. B8381) and incubated for a further 2.5 hr at 37 C. Released sporozoites were not separated from non-

excysted oocysts as dead/non-infective parasite stages were washed from cell monolayers 12 hr after addition to the cultures (Paziewska-Harris et al., 2015). Released sporozoites were used either for infection (see below) or aliquoted for storage in an excess of freezing medium (sporozoites in CMM: storage medium, 1:9). Four different storage media were used: I) RPMI with 20% fetal calf serum (FCS) and 12% glycerol; II) RPMI with 20% FCS and 12% dimethyl sulfoxide (DMSO); III) RPMI with 10% FCS and 5% DMSO (the medium normally used for long term frozen storage of HCT-8 cells); IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO. All media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, provided by Thermo Fisher Scientific, Waltham, Massachusetts).

Oocysts, sporozoites and infected host cells were stored in each of the 4 different media. Samples were cooled to -70 C at the rate of -1 C/min in a mechanical freezer (Mr. Frosty Freezing Container, Thermo Fisher Scientific) before transfer to liquid nitrogen. Two replicates of each combination of *Cryptosporidium* sample and storage medium were stored for each time point (replicates A-B for 6 mo, C-D for 12 mo storage). Thawing after storage in liquid nitrogen was performed at approximately 100 C/min, achieved by 1 min exposure to room temperature followed by 1 min incubation in a water bath at 37 C, a rate compatible with both protozoan survival (e.g., Miyake et al., 2004) and the recovery of host HCT-8 cells. Preparation of different *Cryptosporidium* samples and their processing after freezing was as follows (see also Table I for a summary of study design):

(i) Oocysts (17,300 per replicate) were stored in freezing media for 6 mo. After thawing they were excysted as described above, then divided and the 2 aliquots added to near-confluent HCT-8 cells;

(ii) Oocysts (17,300 per replicate) were excysted and released sporozoites divided into freezing media (4 replicates per medium type). After 6 or 12 mo of storage 2 replicates

stored in each medium were thawed, divided into 2 aliquots and added to fresh HCT-8 cells;

(iii) Newly excysted sporozoites from oocysts pre-treated with trypsin and bile salts as described above (17,300 per replicate) were added to fresh HCT-8 monolayers in 25 cm^2 culture flasks. Infected cells (containing intracellular parasite stages) were harvested on day 3-post-infection and divided between 16 tubes with different freezing media (4 replicates per medium). After storage (6 or 12 mo) they were thawed and each replicate divided and seeded into 2 fresh culture plates containing HCT-8 medium.

For all samples the medium was changed after host cell infection (12 hr post infection) or seeding of infected cells and prior to sampling (12 hr before first sampling). Samples were then collected 3-or 7-days post infection/seeding (d.p.i/d.p.s.); these time points were chosen to allow for any lag in development following freezing. Extracellular parasite stages were recovered from the supernatant (centrifugation at 2,000 *g* for 5 min) and intracellular stages from host cells following trypsin treatment and centrifugation at 2,000 *g* for 5 min. Following centrifugation, pelleted extracellular or intracellular stages were suspended in 950 µl of lysis buffer (Boom et al., 1990).

Additionally, different types of controls were included in the experiment. At the time of freezing, 3 replicates of both intact and pre-treated oocysts were kept as controls to estimate the total number of viable sporozoites and oocysts frozen (control 1). Excystation rate was estimated as described (Paziewska-Harris et al., 2016): after standard excystation using trypsin and bile salts, DNA from parasites was extracted (Boom et al.,1990) and qPCR performed. This method allows estimation of the number of excysted parasites, as DNA is not purified from intact oocysts (Paziewska-Harris et al., 2016). To estimate the quality of excystation and culturing protocols, positive controls were used at every time point (control 2). These were cell monolayers infected with sporozoites excysted from fresh oocysts (less

than 2-mo-old with more than 75% excysting; see Paziewska-Harris et al., 2016). Negative controls (HCT-8 cells without parasites, control 3) were also included. Positive and negative controls were set up and harvested at the same time as experimental samples. To test the differences in infectivity after standard storage in 4 C and storage in liquid nitrogen, oocysts kept for 10 mo at 4 C (control 4) were used to compare with the experimental samples kept at 4 C for 4 mo and then 6 mo in liquid nitrogen. Sub-culturing of cells infected with *Cryptosporidium* was also performed (control 5): HCT-8 cells were infected with sporozoites and sub-cultured 3 days post infection into fresh plates. Samples were then collected on day 3 and day 7 post sub-culturing. To assess the excystation ability of oocysts frozen with different cryoprotectants, triplicates of 1,000 4-mo-old oocysts were stored at -20 C for 1 wk, and after thawing they were subjected to excystation followed by DNA isolation (control 6). Three samples of unfrozen oocysts from the same batch were used as a control.

DNA was isolated as described by (Boom et al., 1990) and qPCR used as rapid method bulk approach to estimate 'zoites' (Paziewska-Harris et al., 2016) rather than semi-quantitative slide-based methods such as immunofluorescence microscopy. This method measures only DNA from living sporozoites as the 12 hr rinse of cultures effectively removes dead sporozoites and DNA released from lysed stages (Paziewska-Harris et al., 2015), giving confidence that only DNA from excysted sporozoites which had remained viable for at least 12 hr was being measured. A standard curve was constructed using a 10-fold dilution series of sporozoites from 20,000 fresh oocysts/µl, which excysted with an efficiency of between 90 to 100% (estimated as in Paziewska-Harris et al., 2016). The highest concentration was therefore assumed to represent 80,000 DNA parasite copies/µl (as each oocyst contains 4 haploid sporozoites). PCR sensitivity was estimated as 0.2 oocyst/µl, which translates to 4 copies of 18S rDNA (as each sporozoite has 5 copies of 18S rDNA gene in the genome; Abrahamsen et al., 2005). At each time point only 2 or 3 samples of each replicate (recovered

151 from different media or controls) were analyzed, precluding statistical analysis and the results
152 are therefore shown as arithmetic means.

153 The mean number of excysting oocysts estimated by qPCR for the 4-mo-old pre-
154 treated input controls (control 1) was 1,540 (of a total of 17,300) per replicate, suggesting a
155 maximum viability for sporozoites of 9%. Positive controls (cells infected with sporozoites
156 from 2 mo-old oocysts with greater than 75% excystation) contained a mean of about 25,000
157 parasite stages after 3 d.p.i. and 18,000 after 7 d.p.i. (control 2). None of the negative controls
158 gave a signal using PCR targeting *Cryptosporidium* DNA (control 3). In all experimental
159 samples and controls the majority of parasite DNA was present in the extracellular phase
160 (data not shown), as also noted by Paziewska-Harris et al. (2015). The results are presented as
161 the total number of parasites recovered, combining both extracellular and intracellular stages.
162 We also confirmed that parasites could be recovered from infected cells harvested on day 3
163 p.i. and sub-cultured to new culture plates (average number of parasites in infected cells
164 while sub-culturing: 275 per sample) (control 5). After 3 days of sub-culturing the mean
165 number of recovered parasites was 320, while after 7 days 310 parasites were recovered.

166 Oocysts stored in liquid nitrogen for 6 mo lost their infectivity regardless of the
167 medium used for storage (see Table II). The total number of *Cryptosporidium* stages
168 recovered from cell cultures infected with sporozoites from 10 mo old oocysts which had
169 been kept in liquid nitrogen for 6 mo did not exceed 50, only 7% and 22% (3 d.p.i. and 7
170 d.p.i., respectively) of the number released from oocysts kept throughout at 4 C (control 4).
171 Previous attempts to freeze oocysts had shown that there is no apparent increase in survival
172 of oocysts frozen with cryoprotectants at -20 C, -80 C or in liquid nitrogen (Fayer et al.,
173 1991; Kim and Healey, 2001). Short-term freezing was more successful (control 6): oocysts
174 frozen for a week in -20 C using different storage media (I-IV) showed viability of 2%-74%
175 as compared to refrigerated controls (maximum of 927 live sporozoites estimated from

oocysts stored in medium II vs. 1,246 from the control samples) (Table II). It appears that the thick oocyst wall prevents cryoprotectants from penetrating the cells, leading to a loss of viability over longer periods at lower temperatures. Parasites stored within host cells also lost viability and only very small numbers (or none) were recovered after 6 and 12 mo storage in liquid nitrogen. Only 1 replicate (cells stored in medium IV for 6 mo and cultured for 7 days) with better parasite recovery from cells than from frozen oocysts (Table II) was noted. This may relate to the small proportion of human cells that recover after freezing.

Excysted sporozoites stored in liquid nitrogen retained viability much better than either oocysts or parasites in host cells; after 6 mo storage those sporozoites could be recovered from all storage media. The highest signal on day 3 p.i. was observed for parasites stored in medium IV, and on day 7 p.i. for those kept in medium III (Fig. 1). The infectivity of sporozoites from oocysts stored at 4 C for 4 mo and then in liquid nitrogen for 6 mo was greater than that of sporozoites from oocysts kept at 4 C for the full 10 mo (control 4). The number of *Cryptosporidium* stages recovered after combination of storage at 4 C and in liquid nitrogen ranged from 1.15× (3 d.p.i.) to 3.3× (7 d.p.i.) the number recovered from cultures using 10-mo-old oocysts stored at 4 C (control 4) (Fig. 2). After 12 mo of storage in liquid nitrogen, the best recovery of parasites 3 and 7 days p.i. came from medium III (Fig. 3), with only few parasites recovered after 12-mo-storage in media I and II. The potential for successful infection by sporozoites stored in liquid nitrogen decreased with time (Fig. 4), but this decline was smallest using medium III; between 45% (7 d.p.i.) and 65% (3 d.p.i.) of sporozoites retained infectivity after 12 mo storage relative to 6 mo storage in this medium. Frozen storage of sporozoites has been tried previously with *Cryptosporidium* (Sherwood et al., 1982; Rossi, 1990; Fayer et al., 1991; Rhee and Park, 1996; Kim and Healey, 2001), but as oocysts did not retain infectivity for neonatal or immunosuppressed mice, the approach was dropped, and it is clear that freezing kills oocysts. Fayer et al. (1991) also observed a

lack of infectivity of rectally-intubated *C. parvum* sporozoites for neonatal BALB/c mice following frozen storage. However, mice are a poor host for *C. parvum*, requiring a much higher inoculum than the 10 oocysts needed to infect calves (Zambriski et al., 2013), and there is no doubt that frozen storage does reduce viability of sporozoites. It may therefore be that in these experiments viability may have been reduced to below the level at which infections could take place. Cryopreservation with simple cryoprotectants may also render sporozoites uninfective in animal models, since the presence of FCS in frozen storage media can potentially trigger the sporozoite-trophozoite transition (Edwinson et al., 2016) prematurely. Nevertheless, we would argue that cryopreservation can provide effective long-term storage medium for sporozoites which is then more consistent than storage of oocysts for equivalent periods at 4 C. Given the overwhelming importance of in vitro studies (e.g., Vinayak et al., 2015; Edwinson et al., 2016; Morada et al., 2016; DeCicco RePass et al., 2017) for understanding the biology of *Cryptosporidium*, and for large scale drug-screening, a potential lack of infectivity of frozen sporozoites in animal models is less significant. Further experiments will certainly fine-tune the method and adapt it for particular experimental needs, but even based on the experimental data provided here it can be concluded that cryopreservation of sporozoites is a viable approach to long-term storage of *Cryptosporidium*. Indeed, even using the methodologies described here, freezing of excysted aliquoted sporozoites is likely to represent a more consistent and possibly more efficient means of keeping isolates than the recommended storage of oocysts at 4 C.

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Figure 1. Number of parasites (mean of 2 replicates) recovered from host cell cultures infected with sporozoites stored in different media for 6 mo. Parasites were harvested 3 days post infection (d.p.i.) (black bars) and 7 d.p.i. (white bars). Storage media used: I) RPMI with 20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10% FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

Figure 2. Differences in proportion of parasites recovered from cultures after storage in liquid nitrogen in different media for 6 mo as compared to controls kept at 4 C. Parasites were harvested 3 days post infection (d.p.i.) (back bars) and 7 d.p.i. (white bars). All the calculations are based on 2 replicates of each condition. Storage media used: I) RPMI with 20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10% FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

Figure 3. Number of parasites (mean of 2 replicates) recovered from host cell cultures infected with sporozoites stored in different media for 12 mo. Parasites were harvested 3 days post infection (d.p.i.) (black bars) and 7 d.p.i. (white bars). Storage media used: I) RPMI with 20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10% FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

Figure 4. Differences in proportion of parasites recovered from host cell cultures infected with sporozoites stored in liquid nitrogen in different media for 12 mo as compared to 6 mo. Parasites were harvested 3 days post infection (d.p.i.) (back bars) and 7 d.p.i. (white bars). All the calculations are based on 2 replicates of each condition. Storage media used: I) RPMI with 20% FCS and 12% glycerol; II) RPMI with 20% FCS and 12% DMSO; III) RPMI with 10% FCS and 5% DMSO; IV) RPMI with 20% FCS, 10% of glycerol and 10% of DMSO.

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Table I. Experimental design: all samples stored in liquid nitrogen for every freezing medium for each time period; d.p.i.- day post infection, d.p.s.- day post seeding.

	Oocysts	Sporozoites	Infected cells
Treatment before storage	None	Excystation	Excystation, host cell infection, harvesting on 3 d.p.i.
Oocysts used per replicate	17300	17300	17300
Total number* of stored parasites per replicate	69200	69200	6920†
Number of replicates	2	4	4
Storage time	6 months (replicates A, B)	6 months (replicates A, B) and 12 months (replicates C, D)	6 months (replicates A, B) and 12 months (replicates C, D)
Treatment after storage	Each replicate excysted, aliquoted (aliquots 1-2)	Each replicate aliquoted (aliquots 1-2)	Each replicate aliquoted (aliquots 1-2)
Culturing	Each aliquot added to fresh HCT-8 monolayer, harvested on 3 d.p.i. (1) or 7 d.p.i. (2)	Each aliquot added to fresh HCT-8 monolayer, harvested on 3 d.p.i. (1) or 7 d.p.i. (2)	Each aliquot seeded on the plate, harvested on 3 d.p.s. (1) or 7 d.p.s. (2)

*Based on 4 sporozoites per oocyst.

†Based on 1% recovery at the time of harvesting infected cells on 3 d.p.i. estimated based on experiments using oocysts of the same age as in the study (data not published).

Table II. Numbers of parasites recovered from cultures after 3 or 7 days post infection/seeding. Oocysts used in the experiments were stored for 4 mo at 4 C. Then either oocysts or HCT-8 cells infected with parasites derived from these oocysts were stored in different freezing media (I-IV, see text for details) in liquid nitrogen (LN; for input numbers see text and Table 1) or at -20 C (1,000 oocysts) for different time periods; d.p.i. - day post infection, d.p.s. - day post seeding; NA- not applicable- only viability was tested.

	Oocysts			Infected cells			
Storage conditions	1 wk at -20 C	6 mo in LN		6 mo in LN		12 mo in LN	
D.p.i./d.p.s.	NA	3	7	3	7	3	7
Medium I	493	16	0	0	0	0	0
Medium II	927	0	29	0	0	6	0
Medium III	147	7	47	0	0	0	0
Medium IV	27	20	8	0	256	42	0

Figure 1

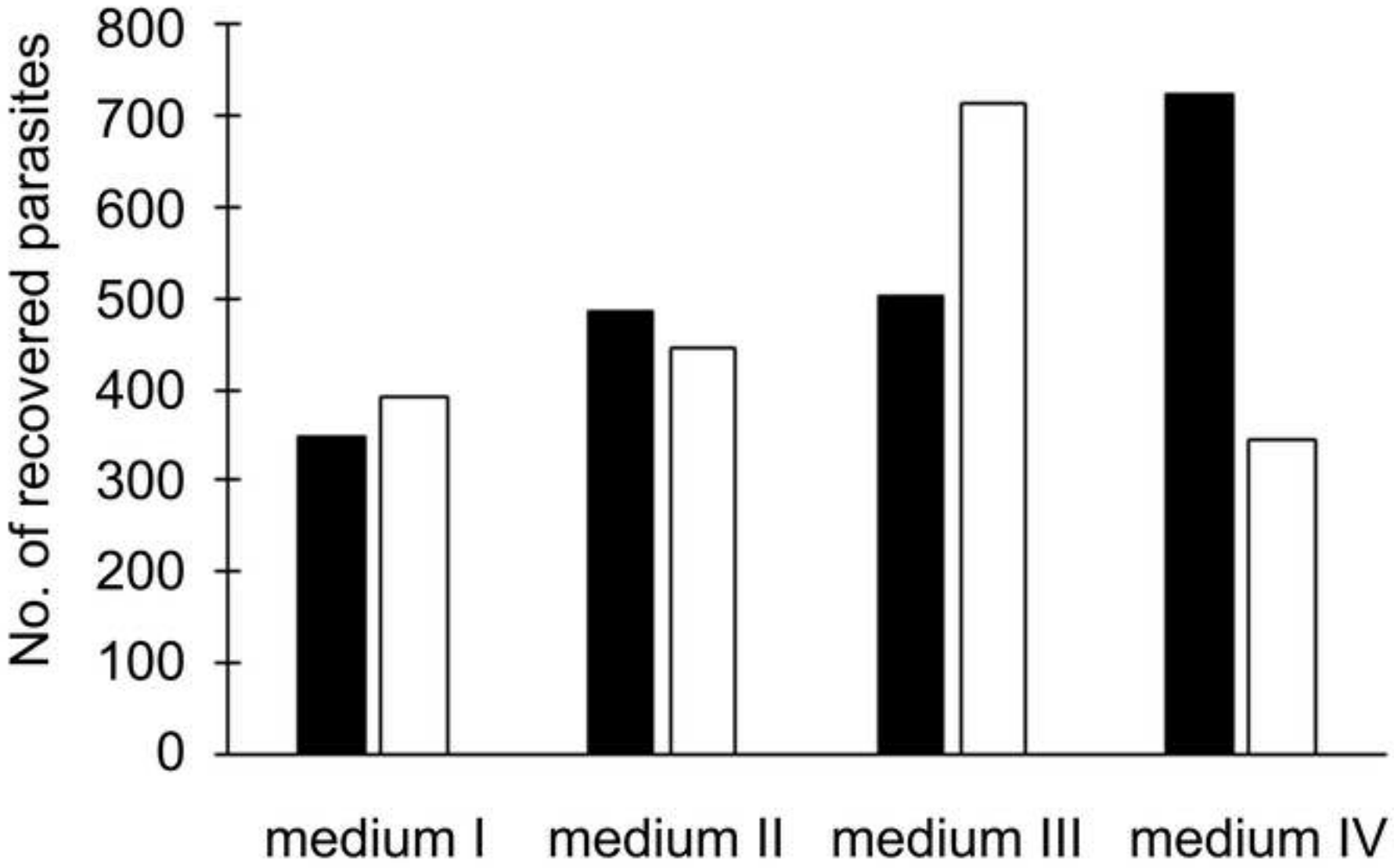

Figure 2

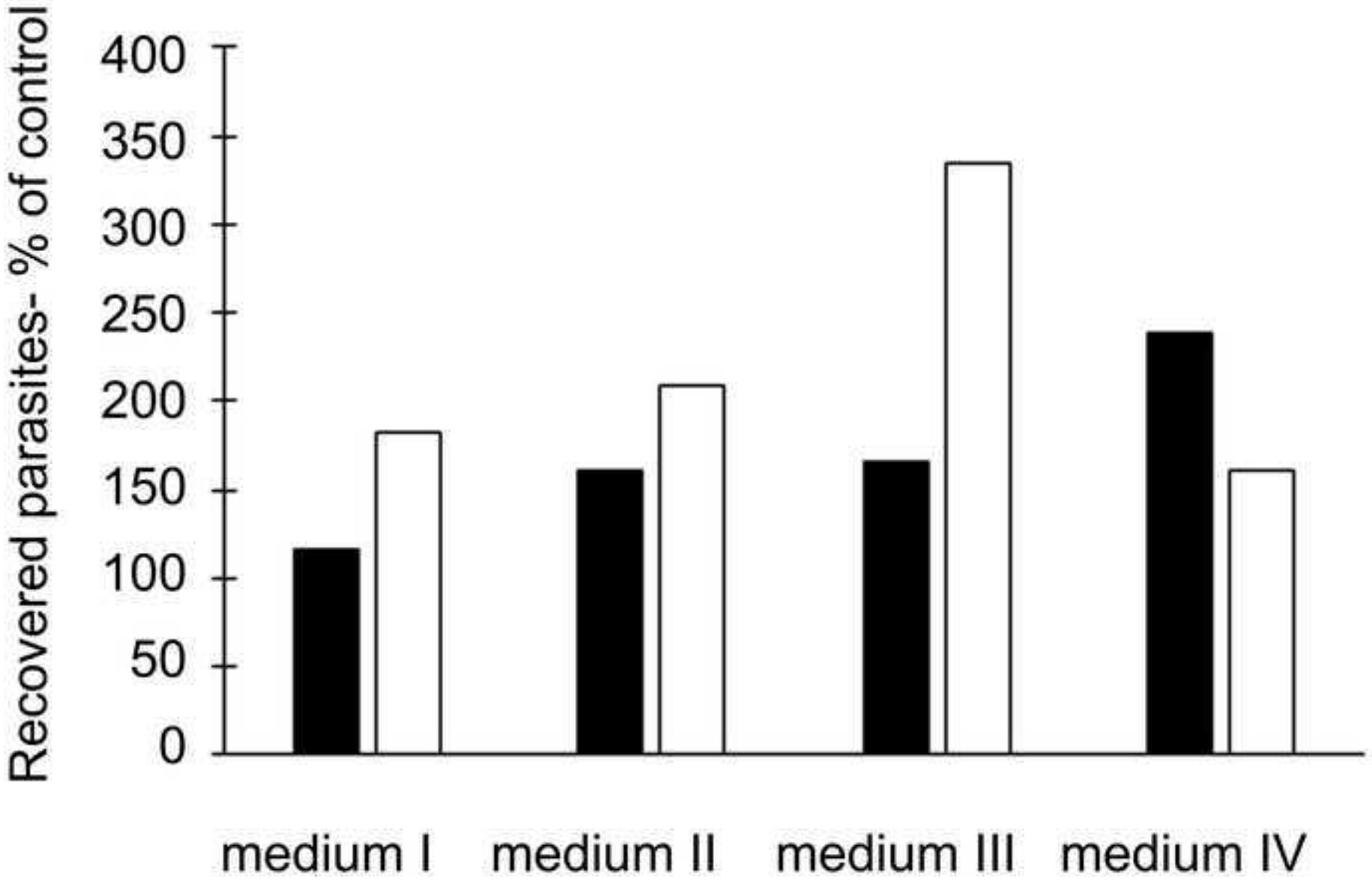

Figure 3

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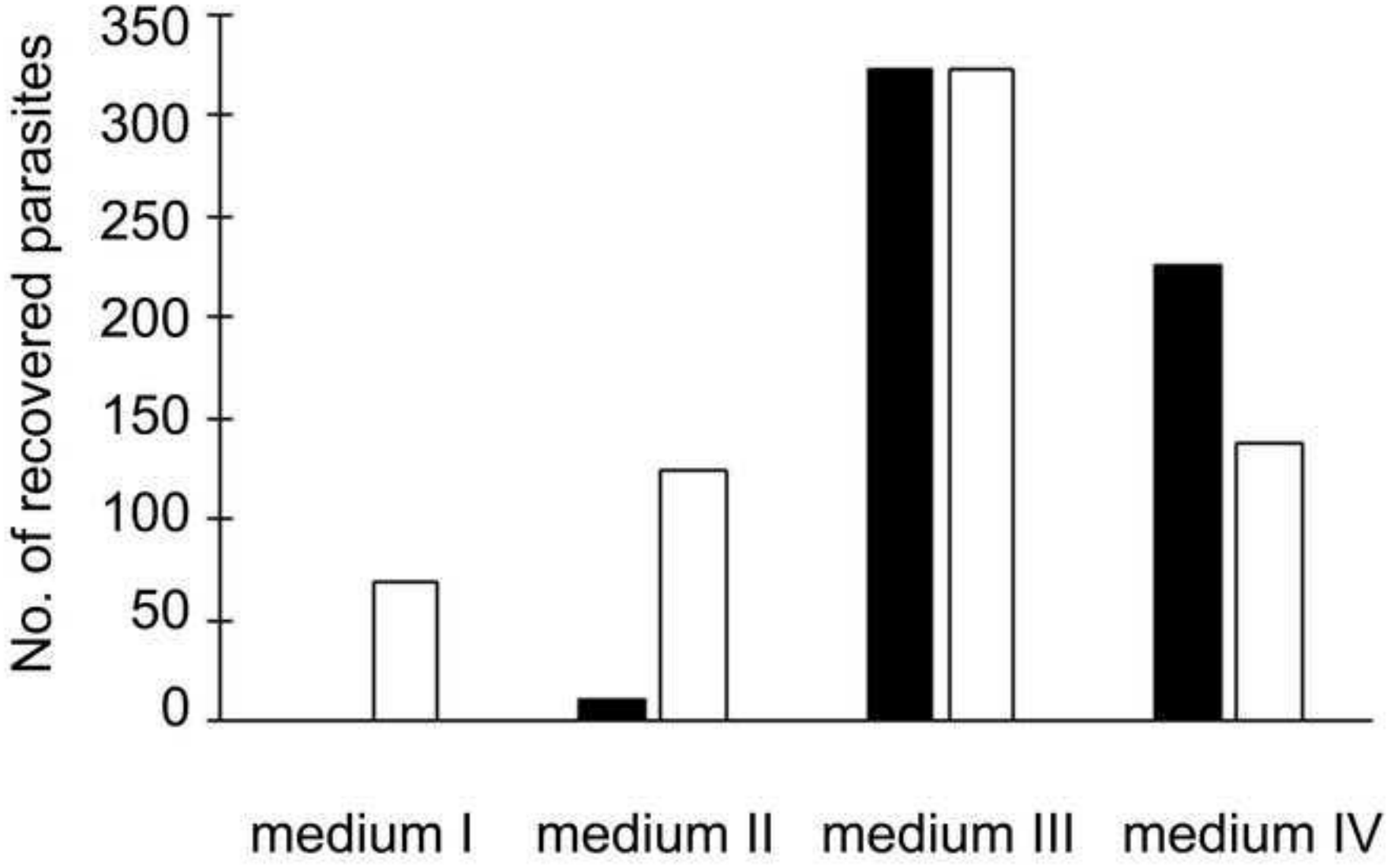

Figure 4